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14. ABSTRACT
The overall goal of this 2 year project was to utilize novel, real-time noninvasive in vivo optical spectroscopic detection methods to investigate potential breakthrough CN poisoning treatment agents, including DMTS alone or with sulfanegen and/or cobinamide, which will be useful for intramuscular (IM) delivery in mass casualty cyanide poisoning settings.

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Introduction:

The overall goal of this 2 year project is to utilize novel, real-time noninvasive in vivo optical spectroscopic detection methods to investigate potential breakthrough CN poisoning treatment agents, including DMTS alone or with sulfanegen and/or cobinamide, which will be useful for intramuscular (IM) delivery in mass casualty cyanide poisoning settings.

Lethal rabbit models of CN poisoning have been developed in our lab. Animals are monitored in real-time for oxy- and deoxy-Hb ratio changes as determined by diffuse optical spectroscopy (DOS) and continuous wave near infrared spectroscopy (CWNIRS), inhaled and exhaled breath gas exchange parameters, blood gas values and blood pressure during CN treatment and reversal.

In the first year of this work, stability and IM antidote administration studies were completed in control cyanide poisoned animals, as well as in animals treated using intramuscular injection of dimethyl trisulfate (DMTS) as the CN antidote (one of the 3 novel therapeutic agents under development with our collaborators). DMTS was formulated in a 15% polysorbate vehicle solution (which was found to be stable for > 1 month). The DMTS/polysorbate agent was successfully administered by intramuscular injection in 12 animals. Survival in the lethal model increased from < 20% in controls to 75% survival in DMTS treated animals. The rate and extent of acute poisoning and reversal in the periphery and CNS region was monitored noninvasively using these optical technologies concurrently with the systemic measurements.

During the second year of the project, the minimal effective dose ranges for DMTS and for cobinamide were determined individually, and testing of a variety of combinations of the agents was completed.

Body:

Scope of Work: The scope of work for this 2-year effort includes the specific tasks from the original proposal described herein:

1). Investigate the ability of DMTS to be absorbed and reverse cyanide poisoning, and optimize in vivo lipid based delivery in the animal model by intramuscular injection. As part of this process, the contractor will develop noninvasive methods for measuring DMTS absorption/uptake in real-time using DOS. Contractor will utilize novel real-time noninvasive in vivo optical (DOS) detection methods to investigate potential breakthrough treatment agents (DMTS alone or with sulfanegen and/or cobinamide) for mass casualty cyanide poisoning that can be administered intramuscularly (1-4).

Status: completed.

Progress: A series of dose response experiments using DMTS formulations were carried out. These experiments were successful in determining a dose range over which DMTS appeared to be an effective antidote, but initially, inconsistencies were observed during the series of experiments. In particular, it was noted that older DMTS solutions were not as effective as new formulations. In conjunction with Drs. Gary Rockwood and Ilona Petricovics, significant loss of DMTS in the polysorbate solution was verified by GC-MS. Studies were initiated to examine the stability of DMTS formulations over time and in various conditions of storage. Experiments revealed that DMTS in polysorbate was stable for 60+ days if stored in airtight, double-sealed vials at 4^{o}C. Single-use samples were subsequently provided to us for further experiments, and periodic verification of stability is now carried out by Dr. Petricovics via GC-MS.

Following the completion of stability testing, we were able to complete a series of experiments in the ventilated, lethal rabbit CN model, which verified the efficacy of DMTS injected IM in a lethal, ventilated, cyanide poisoned animal model system. The lethal ventilated model experimental timeline is shown in Figure 1, below, and described in detail in the Supporting Data section below.

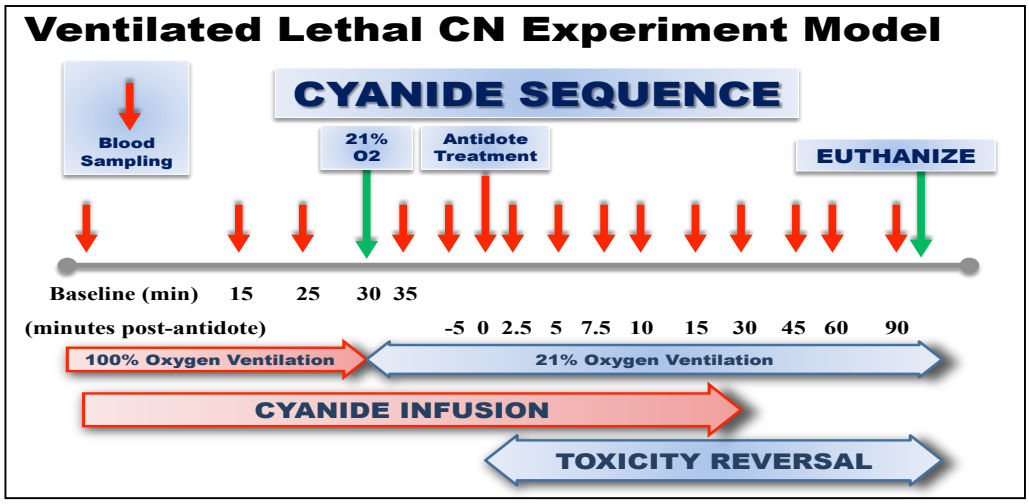

Figure 1: figure 1 shows the study design for lethal cyanide poisoning rabbit model DMTS antidote efficacy investigations. Animals are monitored at baseline. A continuous cyanide infusion is begun. Animals are monitored continuously with CWNIRS and DOS optical technologies, as well as invasive arterial and venous lines, and inhaled and exhaled gas analysis. Animals are given intramuscular antidote injection (or control vehicle injection) when they develop signs of impending lethal CN toxicity (criteria as described in detail in appendix).

Results:
75% (9 of 12) of the animals treated with 150mg of DMTS in Polysorbate survived the experimental protocol to the 90-minute post-antidote time point. This is in comparison to a 20% survival in cyanide-poisoned animals treated with placebo injection.

2. Evaluate the effectiveness of combined DMTS with cobinamide and sulfanegen.

Experiments to investigate the effectiveness of DMTS when combined with cobinamide were completed. The submitted project timeline included the completion of these experiments as a Year 2 task. The first task in combination of the 2 agents was to titrate the threshold doses of DMTS, cobinamide and sulfanegen alone in the lethal, ventilated model, such that survival with individual antidotes was <50%. This was accomplished, and followed by a series of combination experiments, as shown in Figure 2.

Analysis of these experiments indicated that the DMTS in combination with cobinamide appears to be a highly effective combination, and additive at doses below their individual efficacy. More experiments would be necessary to prove synergy. The combination of tetranitrocobinamide and magnesium or sodium thiosulfate is a highly effective CN antidote, allowing for a small injection volume IM antidote to be developed.

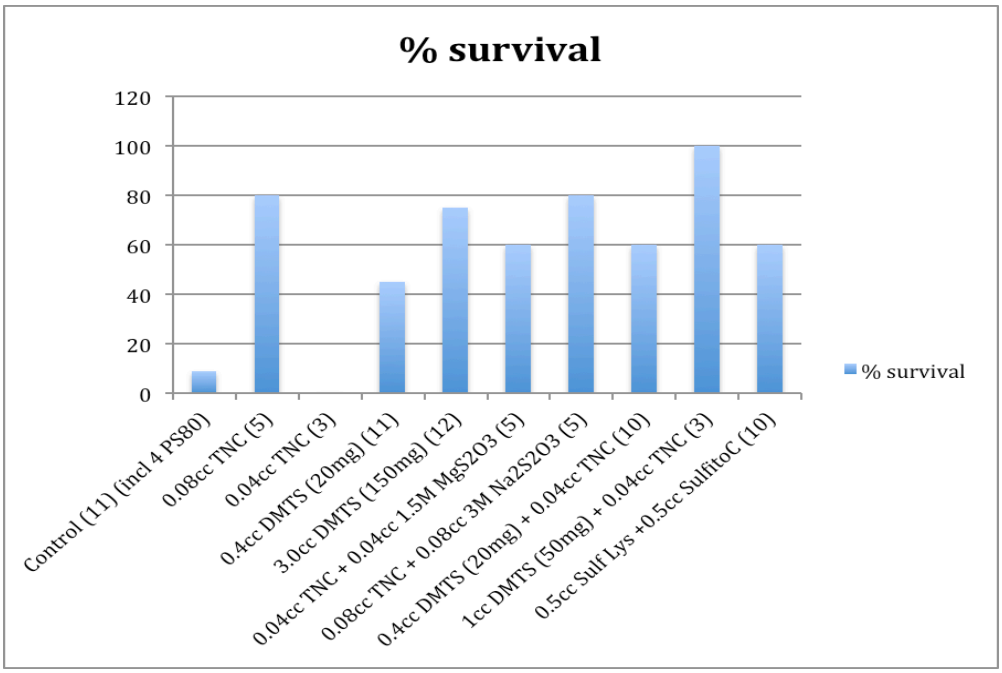

Figure 2: Percent survival in combinations of DMTS, tetranitrocobinamide (TNC), sulfitocobinamide (SulfitoC) and sulfanegen lysine (Sulf Lys). Numbers in parentheses indicate animals /group.

3. Assess the effects of these novel antidote agents on CNS region oxygenation and CNS metabolic response using noninvasive/minimally invasive functional optical brain monitoring and imaging technologies.

Continuous wave near infrared spectroscopy (CWNIRS) has been used in all DMTS experiments to date to follow and compare the oxy and deoxyhemoglobin changes and time course in response to CN poisoning and antidote treatment in the brain region as compared to muscle tissue. Figure 3 shows the results of a control experiment, in which animals received cyanide infusion, but no antidote. Comparing CWNIRS plots of oxy and deoxyhemoglobin from the CNS and muscle regions, marked differences are seen in the physiologic effects of cyanide poisoning in CNS regions compared to periphery. As cyanide poisoning progresses, cyanide inhibits cytochrome C oxidase, blocking aerobic metabolism and the ability of tissues to extract oxygen from the circulating blood stream. This is manifested by increases in oxyhemoglobin (red curves) and decreases in deoxyhemoglobin (blue curves). Terminally, cardiovascular collapse ensues and oxyhemoglobin plummets in the final stages (figure 2). Because of the high blood flow, autoregulation, high metabolic needs, and inability to utilize anaerobic metabolism, the CNS changes are more profound and occur much more rapidly than the peripheral muscle effects.

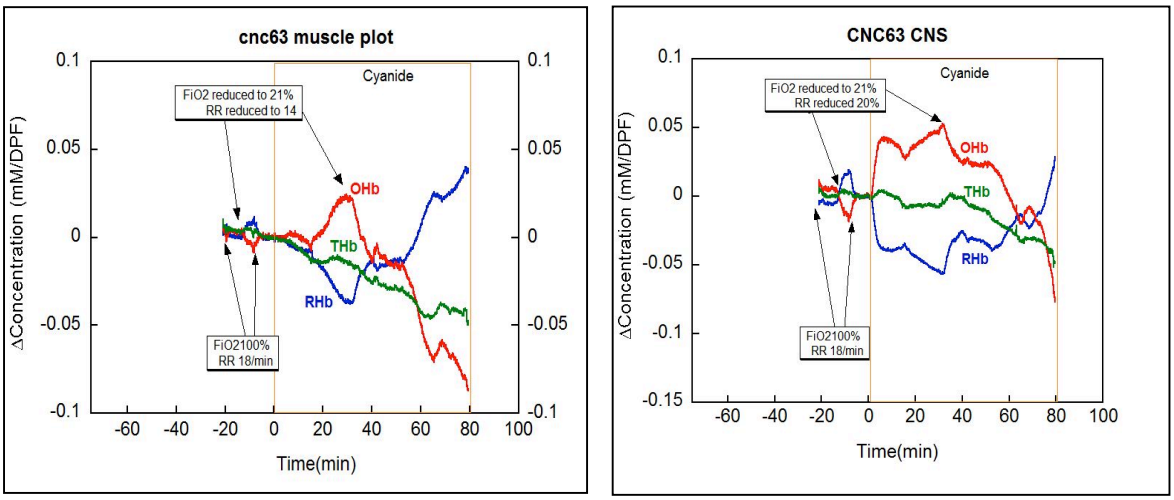

Figure 3. Lethal ventilated cyanide experiment control saline treatment, showing the CWNIRS measured oxy hemoglobin (red, OHb), deoxy hemoglobin (blue, RHb), and green, total hemoglobin during cyanide infusion and saline injection. Saline, given at 30 mins of NaCN infusion, did not reverse the effects of fatal CN poisoning. The CNS/brain region typically shows the hemoglobin effects of cyanide poisoning earlier than the peripheral muscle tissue.

In contrast, when animals are given IM DMTS antidote injection as shown in figure 4, the oxyhemoglobin and deoxyhemoglobin changes are blunted, and the terminal deterioration is prevented.

Figure 4 illustrates the effects of reversal of lethal cyanide poisoning with IM injection of DMTS as monitored noninvasively using DOS and CWNIRS in the peripheral muscle and CNS regions.

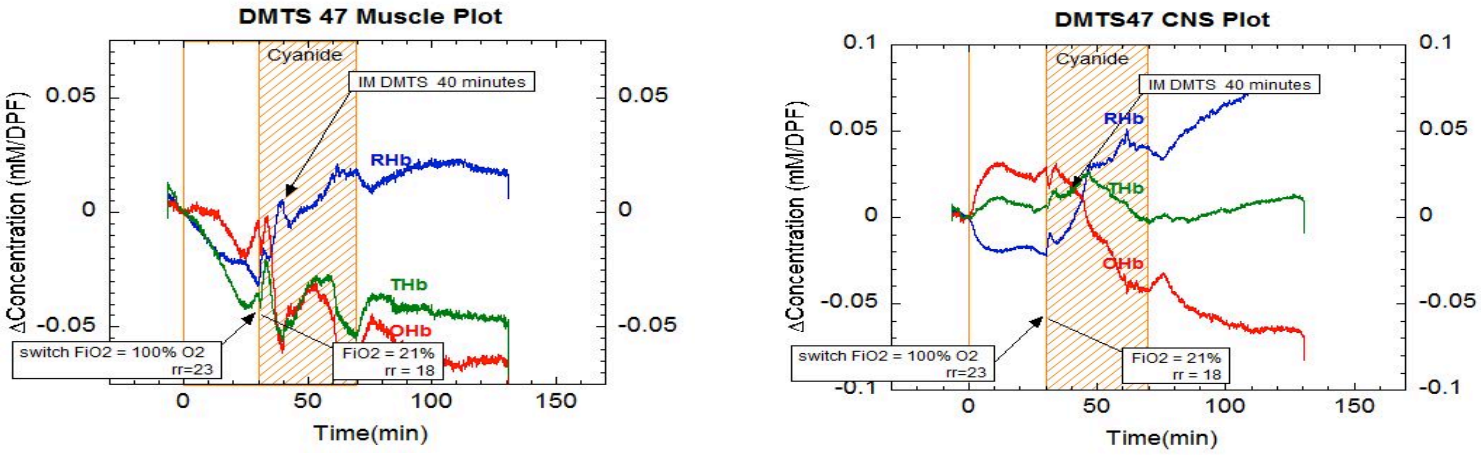

Figure 4: in this figure, animals received continuous cyanide infusion, which results in inability of tissues to extract oxygen. This is manifested by the rise in CNS oxyhemoglobin and decrease in deoxyhemoglobin (right-sided figure). When IM DMTS is administered at 40 minutes into the cyanide infusion, these changes are rapidly reversed and lethality is prevented. In the peripheral muscle region, a decrease in total hemoglobin (green line) is seen that is not present in the CNS due to autoregulation. This decrease in total hemoglobin (secondary to reduced muscle blood flow) accounts in part for the lack of rise of oxyhemoglobin.

These studies confirm the capabilities of DOS and CWNIRS to noninvasively monitor the progression of cyanide poisoning and reversal and effectiveness of antidote therapy.

4. Use a mechanistic research approach to identify targets for therapeutic development using novel in vivo optical signal detection approaches.

This task is part of the overall direction of our laboratory and the collaborative IAA/CounterACT research programs. We continue to examine various metabolic parameters including oxy and deoxyhemoglobin, inhaled and exhaled gas exchange (anaerobic threshold), hemodynamics, and optically determine to cytochrome C oxidase redox state potential to understand the mechanisms of cyanide poisoning on a physiologic level in vivo. Experiments analyzed have demonstrated that cytochrome C oxidase redox state signals can be determined in vivo (independent from oxyhemoglobin and myoglobin optical cross talk signals), and that significant reduction in cytochrome C oxidase occurs before respiratory exchange ratios increase and anaerobic metabolism ensues. Reversal of cyanide poisoning results in return of respiratory exchange ratios and improvement in cytochrome C oxidase redox state. In figure 5, typical examples in surviving animals show the return of cytochrome c oxidase redox state to nearly baseline levels after high dose DMTS versus low dose DMTS. Even at low dose (50% survival for animals given 20 mg DMTS), improvement in cyt c oxidase redox state is seen.

These findings will be important in understanding metabolic agent poisoning, providing ongoing means for quantitative determination of treatment efficacy and resuscitation.

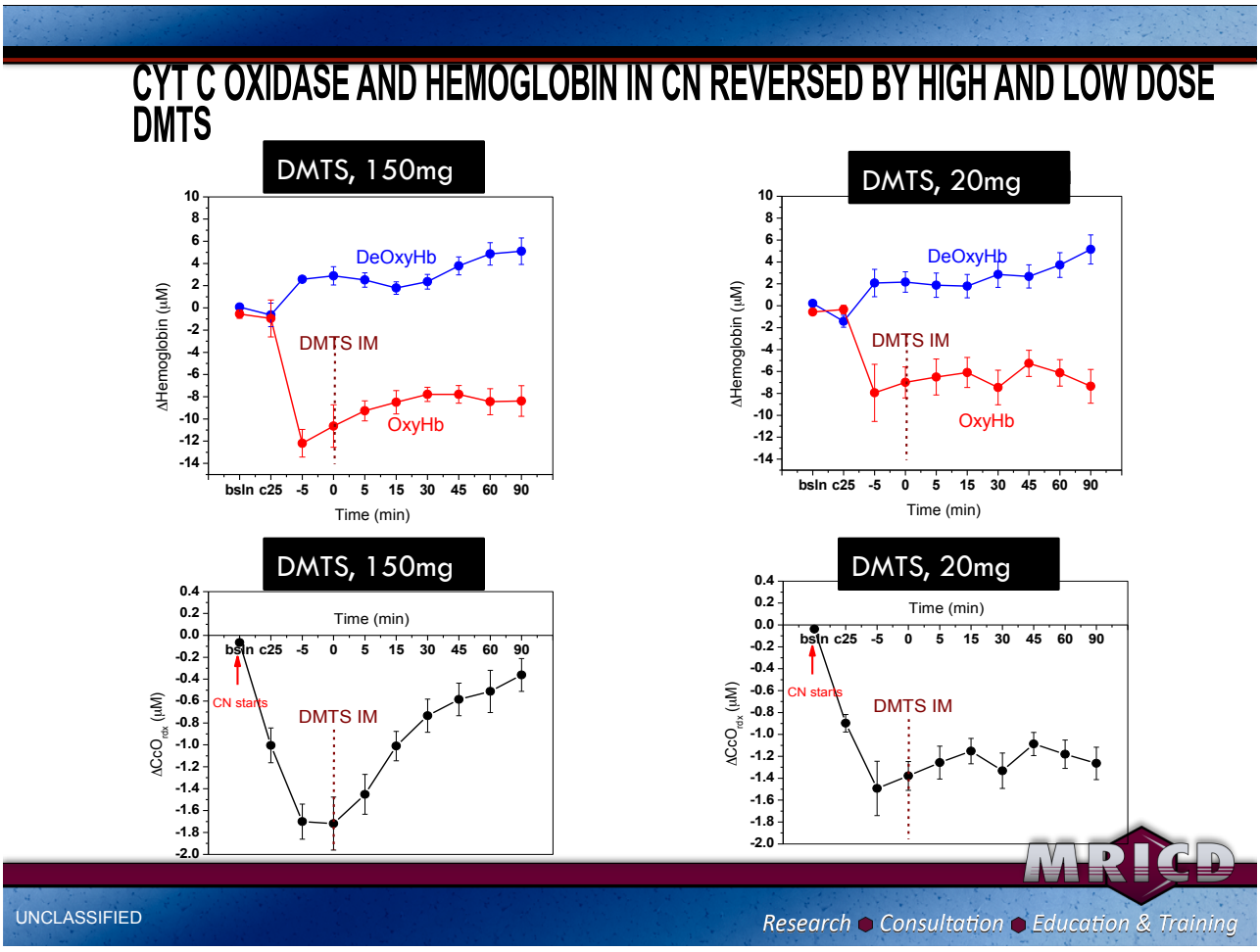

Figure 5: Experiments using DOS to analyze the oxy and deoxyhemoglobin concentrations and the cytochrome c oxidase redox state return following high dose DMTS administration versus low dose DMTS administration. High dose, N=12, 9/12 animals survive. Low dose, N=11 5/11 animals survive. Average animal weight is 4 kg. Surviving animals typically receive 23-28mg NaCN. These examples in surviving animals show the return of cytochrome c oxidase redox state to nearly baseline levels after high dose DMTS

5. Perform creation and validation of animal models of chemical effects on humans using novel rabbit poisoning models.

No animal model can perfectly reproduce the human response. Furthermore, human CN exposure scenarios will differ widely. Therefore, numerous animal models must be developed to answer a range of aspects of exposures and treatments. The importance of this issue is further compounded by the fact that human efficacy testing cannot be performed prior to FDA approval for CN antidotes. However, fortunately for metabolic poisons that affect very basic aerobic metabolism pathways, substantial similarities do exist between mammalian species and humans. We have developed a number of rabbit model variations including sublethal models, lethal models, ventilated models, nonventilated models, and IV oral cyanide exposure models. We have administered antidotes via intramuscular injection, inhalation, and intraosseous routes. These animal models are all available for ongoing testing of the novel candidate antidotes alone and in combination as described in the proposal. Work is ongoing.

6. Perform development of preliminary proof of principal data on the efficacy of candidate therapeutics with potential treatment breakthrough agents DMTS, sulfanegen, and cobinamide.

Experiments have been completed that demonstrate the efficacy of each of these agents by intramuscular administration as single and combined agents in the rabbit model. These studies have demonstrated evidence of effectiveness of DMTS with cobinamide. These studies indicate the additive effects, and additional studies will still be needed to determine any synergy in the agent combinations. Combined DMTS and cobinamide therapy appeared to be effective and additive, and further development and optimization of ratio and co-formulation would be valuable. Synergy has not yet been demonstrated for the 2 drugs, but the smaller doses of individual drugs, possibly minimizing individual drug side effects, may be valuable when formulating antidotes for human use.

7. Perform development of novel optical methods for determining the rate of antidote absorption in vivo in brain and peripheral regions using diffuse optical spectroscopy and functional optical monitoring.

Continuous wave near infrared spectroscopy is used in all rabbit experiments to examine the changes in oxy and deoxyhemoglobin ratios in brain and muscle tissue. A comparison of the responses of brain versus muscle has demonstrated increased sensitivity of the brain to cyanide poisoning and reversal in comparison to periphery. This is likely secondary to the multiple factors in the CNS described above including metabolic requirements, inability of CNS to utilize anaerobic pathways, high blood flow rates, and autoregulation.

Key Research Accomplishments:

Task 1:

- Developed stabilized formulations of DMTS polysorbate and methods for storage and transport.

- Demonstrated effectiveness of intramuscular injection of DMTS in reversing lethal cyanide poisoning in the rabbit model.
- Utilized DOS and CWNIRS concurrently with systemic monitoring to determine rates and extent of reversal of cyanide poisoning.

Task 2:

- Demonstrated effectiveness of combined therapy with DMTS and cobinamide.

Task 3:

- The CNS and peripheral effects of cyanide poisoning were shown using DOS and CWNIRS. These studies confirm the capabilities of DOS and CWNIRS to noninvasively monitor the progression of cyanide poisoning and reversal and effectiveness of antidote therapy.

Task 4:

- Reversal of cyanide poisoning results in return of respiratory exchange ratios and improvement in cytochrome C oxidase redox state. These findings will be important in understanding metabolic agent poisoning, providing ongoing means for quantitative determination of treatment efficacy and resuscitation.

Task 5:

- We have developed a number of rabbit model variations including sublethal models, lethal models, ventilated models, nonventilated models and oral cyanide exposure models. We have administered antidotes via intramuscular injection, inhalation, and intraosseous routes. These animal models are all available for ongoing testing of the novel candidate antidotes as was completed in the studies here, and for future studies.

Task 6:

- Experiments have been completed that demonstrate the efficacy of each of these agents by intramuscular administration as single agents in the rabbit model. The combination of DMTS and cobinamide appeared additive and effective in this model. The combination of tetranitrocobinamide and thiosulfate appeared to be highly effective as a cyanide antidote, and allowed for very small and easily scalable IM injection volumes for human use.

Task 7:

- Differences between CNS and peripheral responses to CN toxicity and reversal have been observed and described using DOS and CWNIRS technologies.

Deliverables:

1. Demonstrate improved outcome in CN poisoning with DMTS.

Complete Groups 1-3 studies with these endpoints and success criteria:

Endpoints: Primary 1) Survival. Secondary: 1) systemic blood pressure improvement, 2) tissue CcO redox state and systemic gas exchange/hemodynamic improvement.

Success Criteria: Primary: 1) improved survival.

Secondary: 1) statistically significant increase in blood pressure in hypotensive poisoned animals, 2) return of cytochrome c oxidase redox states towards pre-poisoned baseline with gas exchange measures showing improved respiratory quotient.

The primary endpoints of success criteria have been met for DMTS intramuscular treatment of lethal cyanide poisoning in the animal model. Statistically and clinically significant increase in survival from 15% to greater than 70% has been obtained compared to control cyanide poisoned and treated animals. With these dramatic improvements in survival, all of the secondary criteria have also been demonstrated (no terminal blood pressure drops, metabolic, or DOS/CWNIRS terminal phase changes in the surviving treatment group animals that were seen in all of the expiring control group animals.

Combined DMTS and cobinamide therapy appeared to be effective and additive, and further development and optimization of ratio and co-formulation would be valuable. Synergy has not yet been demonstrated for the 2 drugs, but the smaller doses of individual drugs, possibly minimizing individual drug side effects, may be valuable when formulating antidotes for human use.

Reportable Outcomes: (pubs, abstracts, patents, etc.)
These results have been presented at the CounterACT meetings in Washington DC June, 2013, and in Denver, CO June, 2014. Manuscripts are being prepared at this time. Publication in open meetings and journals is being held (at the request of ICD) until patent rights issues have been cleared by the Department of Defense/ICD allowing us to release findings into the public domain.

Conclusions:
The work supported in this proposal has contributed to the development of a highly stable and effective formulation of DMTS. The work has demonstrated the efficacy of DMTS as an IM antidote over a range of doses in a lethal rabbit model of CN toxicity. Studies have revealed the additive ability of DMTS and cobinamide to reverse lethal CN toxicity in the model, and have demonstrated the effectiveness of a combination of tetranitrocobinamide with thiosulfate. The unique DOS and CWNIRS technologies have provided valuable information on real-time changes in oxy-and deoxyhemoglobin, and DOS can provide information on the efficacy of CN reversal agents by tracking the reversal of CN effects on CcO redox states.

References: N/A
Appendices:

Detailed procedure for the ventilated, lethal CN rabbit model:
All animals are anesthetized, intubated, and femoral venous and arterial lines placed. After the placement of the femoral catheters, baseline measurements (venous and arterial blood gases, systemic blood pressures, heart rate and SpO2 values) are taken. The non-invasive DOS (over muscle) and CWNIRS (1 on muscle, 1 over the brain region) monitors are placed. In some animals, the Botvinick microlactate sensor is introduced into the dermal layer of the animal using a 26g needle to provide lactate level measurements. During baseline measurements, the ventilator settings are maintained at a typical respiratory rate of 20 breaths/min, with the inhalation gas input of 100% oxygen.
A pediatric pressure and gas sensor of a GE/Datex Ohmeda S5 (S5) Anesthesia Monitor (with metabolic module, M-COVX) is placed between the “Y” of the ventilator tubing and ET tube connector. After optical measuring devices are connected, the rate of IV anesthesia is increased from .17 to .19 cc/min at least 10 minutes of metabolic data are collected using the S5 equipped with the pediatric flow/pressure pneumotach (data is recorded once a minute on spreadsheet). The S5 can measure oxygen consumption (VO2) in mls/min, carbon dioxide production (VCO2) in mls/min, respiratory rate (rr) in breaths /min, inhaled fraction of oxygen (FiO2) and CO2 (FiCO2), end tidal fraction of oxygen (FiO2 – FetO2) and CO2 (FetCO2), inhaled and exhaled minute volumes (mls/min). After baseline values are taken by the DOS, CWNIRS and S5 with the inhaled fraction of oxygen set to 100% (the S5 device cannot calculate VO2 when inhaled oxygen is set to 100%), the inhaled oxygen delivered from the ventilator is reduced to 21% (subject breathing room air). Measurements are recorded from the S5 every minute for roughly 10 minutes. After 10 minutes of breathing room air, the ventilator is reset to 100% oxygen and cyanide infusion begins after the effects of inhaling room air are reversed.
Cyanide Infusion:
20 mg of sodium cyanide dissolved in 60 ml of 0.9% NaCl saline is infused intravenously at .33 mg/min (1cc/min). Cyanide is continuously infused for 30 minutes with 100% oxygen input from the ventilator. During cyanide infusion, measurements from the S5 continue to be collected every minute. Time point measurements, which include arterial and venous blood gases, systemic pressures, heart rate, and SPO2 are taken at time intervals: 15 min after cyanide infusion has started, 25 minutes, and 35 minutes, and continuous DOS and CWNIRS monitoring occurs. At the 30-minute post cyanide

infusion time point, the ventilator inlet source is changed from 100% O2 to room air, and the ventilator setting is adjusted to 16 breaths per minute. The rabbit is then monitored to determine when the cyanide infusion dose has become lethal and when the experimental treatment should be administered. This determination is made as follows:

The effects of cyanide toxicity can be observed in real time, and include a reduction in blood pressure, oxygen consumption, CO2 production (as well as the fraction of exhaled O2 and CO2), and the CWNIRS curves that show changes in the ratios of deoxy HGb and oxy HGb in real time.

The “minus 5 minute” (5 minutes before IM or IV cyanide antidote injection) time point is determined by a reduction of VO2 clearly over 50% from baseline, a significant reduction (>20% from baseline) VCO2, a fall in mean blood pressure 40% from baseline, and the DOS and CWNIRS indicating an acceleration of the effects of cyanide poisoning (increased slope changes in deoxy HGb and oxy HGb). After the “minus 5 minute” blood samples are taken, the experimental treatments are prepared. At time point “0”, each animal will then receive the cyanide reversing drug treatment DMTS (or vehicle control). The treatment is administered via intramuscular injection into the (pectoral muscle), via intraosseous injection into the tibia, or nebulized using a DeVilbiss ultrasonic nebulizer. Following the injections, blood pressures are monitored every minute and noted for the first ten minutes. Cyanide level and blood gas samples are taken at times 0 min, 2.5 min, 5 min, 7.5 min, 10 min, 15 min, 30 min, 45 min, 60 min, and 90 minutes post treatment. Cyanide infusion continues for 30 minutes post treatment. Time point measurements, including arterial and venous blood gas, heart rate, blood pressure and SPO2 are also taken at time 5 min, 15 min, 30 min, 45 min, 60 min, and 90 minutes. At the 90-minute time point, final measurements are taken, and the rabbit is euthanized. If at any time during the experiment, the systolic blood pressure falls to 75% below the baseline value, or25mmHg systolic, (this occurs in > 80% of the control animals receiving no antidote treatment), the procedure will be considered “lethal”, the experiment will be stopped, and the animal are euthanized immediately.